METHOD OF PRODUCING AND PURIFYING ANGIOSTATIN PROTEIN

FIELD OF THE PRESENT INVENTION

The present invention relates to a novel method of recombinantly producing, recovering and purifying angiostatin protein (EntreMed Inc., Rockville, MD).

BACKGROUND OF THE INVENTION

Angiostatin is a protein which is a potent inhibitor of developing blood vessels and tumor growth. Angiostatin is believed to play an important role in the inhibition of the development of blood vessels to new tumor metastases.

Isolation and purification of proteins, such as angiostatin protein, in high yield from biological material, such as tissue extracts, cell extracts, broth from incubation systems, and culture medium is often frought with problems in view of the numerous proteins and other undesirable molecules present in an homogenate or extract. What is needed are recombinant methods of producing angiostatin protein that will provide the large amounts of angiostatin protein required for clinical use, including, but not limited to, cancer therapy. Such methods should produce angiostatin protein in an efficient and convenient manner in a culture broth which is amenable to procedures designed to recover and purify angiostatin protein in high yields. Separating a, specific protein of interest from potential contaminants presents a challenge in view of numerous factors, such as contamination of cellular homogenates with proteolytic enzymes that may digest the protein. Other undesirable cellular constituents that may be present in homogenates, include but not limited to, pigments, cytochromes, lipids, free radicals, oxidases and other lysosomal enzymes, and oxides. Some of these substances may affect the protein of interest by stripping electrons, affecting disulfide bonds and changing the conformation of the protein.

Centrifugation of cells, including yeast, bacteria, insect and other cells used for recombinant production of proteins, such as angiostatin protein, could possibly result in damage to the cells with concomitant release of undesirable biological material. What is needed is a method for recovery and purification of protein, such as angiostatin protein, which does not employ centrifugation.

Methods for recombinant production, recovery and purification of angiostatin protein on a large scale are required to produce and isolate the amounts of purified angiostatin protein needed for administration to patients and also for research purposes.

Also needed is a method for purifying recombinantly-produced angiostatin protein which avoids the need for centrifugation of the culture broth, thereby avoiding problems associated with cell lysis. This method should be capable of use on a large scale to recover and purify angiostatin protein in quantities needed for clinical administration and research.

What is also needed is a method for purifying recombinantly produced angiostatin protein which minimizes contamination with cytochromes, pigments, enzymes, and other undesirable cellular constituents.

Also needed are solutions for storage of angiostatin protein following the recovery and purification process which optimizes solubility properties of angiostatin protein.

SUMMARY OF THE INVENTION

The present invention solves .these problems inherent in the recovery and purification of proteins, particularly angiostatin protein, by providing new and useful methods for recombinant production, recovery and purification of proteins, especially angiostatin protein. The present invention provides new and useful methods for recombinantly producing angiostatin protein in large amounts. The present invention provides a method for recovery and purification of angiostatin protein. The present invention also provides new

and useful solutions for storage of angiostatin protein. These methods provide the benefit of preserving the biological activity of angiostatin protein. Preservation of the biological activity of angiostatin protein is crucial for administration of angiostatin protein to humans and animals for the purpose of inhibition of undesirable angiogenesis, for other biological activities, and for research investigations or other types of biological testing.

Angiostatin protein is effective in treating diseases or processes that are mediated by, or involve, angiogenesis. The angiogenesis mediated diseases include, but are not limited to, solid tumors; blood born tumors such as solid tumors, blood borne tumors, leukemias; tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, colon cancer, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation.

In one embodiment, the present invention provides new and improved methods for recombinant production of biologically active angiostatin protein in high yield.

In another embodiment, the method of the present invention is useful for recovery and purification of recombinantly-produced angiostatin protein.

In another embodiment, the method of the present invention is useful for recovery and purification of angiostatin protein from extracts of biological fluids, cells and tissues.

An advantage of the present invention is that higher amounts of biologically active angiostatin protein are recombinantly produced. Another advantage of the present invention is that greater amounts of angiostatin protein are recovered than obtained with prior art methods. Yet another advantage of the

present invention is that higher yields of more purified, and biologically active angiostatin protein are obtained. Still another advantage of the present invention is that angiostatin protein may be stored in buffers for extended periods of time, and also subjected to lyophilization, while preserving biological activity. An advantage of the present invention is that it permits angiostatin protein to be stored in vials or other containers, either in a solution which may be liquid or frozen, or lyophilized, and optionally shipped to a recipient.

Accordingly, an object of the present invention is to provide an improved method for recombinant production of large amounts of biologically active angiostatin protein.

Another object of the present invention is to provide a method for recovery and purification of recombinantly produced proteins.

Yet another object of the present invention is to provide a method for recovery and purification of angiostatin protein.

Another object of the present invention is to provide a method for recovery and purification of angiostatin protein, particularly recombinantly produced angiostatin protein.

An advantage of the purification methods of the present invention is that undesirable proteins, lipids and pigments are efficiently separated from the desired protein, especially angiostatin protein.

It is another object of the present invention to provide solutions which provide favorable solubility conditions for angiostatin protein, particularly recombinantly-produced angiostatin protein while retaining biological activity of angiostatin protein.

Another advantage of the methods of the present invention is that centrifugation of the broth from fermentation steps in recombinant production of angiostatin protein is avoided, thereby preventing unwanted cellular lysis and potential contamination of angiostatin protein with additional proteins, pigments, enzymes and other cellular chemicals and debris.

Another object of the present invention is to provide methods amenable to large scale production, recovery and purification of recombinantly-produced angiostatin protein.

Another advantage of the present invention is that the recovered and purified angiostatin protein is provided in a solution which optimizes solubility of angiostatin protein, while preserving the bioactivity of angiostatin protein.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a process overview diagram for the large scale processing of purified angiostatin protein.

Figure 2 is a process flow diagram for fermentation inoculum preparation for angiostatin production.

Figure 3 is a process flow diagram for fermentation and streamline SP chromatography for angiostatin production.

Figure 4 is a process flow diagram for chromatography steps following streamline SP chromatography for angiostatin production.

Figure 5 is a process flow diagram for chromatography, ultrafiltration, diafiltration and formulated bulk processing steps following Toyopearl chromatography for angiostatin production.

DETAILED DESCRIPTION

Angiostatin is a protein which is a potent inhibitor of developing blood vessels and tumor growth. Angiostatin is believed to play an important role in the inhibition of the development of blood vessels to new tumor metastases. The following pages of this patent application describe new procedures and protocols for the large scale production of human recombinant angiostatin from Pichia pastoris fermentation of clones with nucleic acid sequences encoding for

angiostatin protein or variants thereof. This application also provides new procedures for the large scale production, purification, characterization and storage of human recombinant angiostatin. It is to be understood that the methods of the present invention are not limited to human recombinant angiostatin, and that the present methods apply to angiostatin from other species, as well as fragments and conservatively substituted forms thereof.

The method can serve as a large scale purification protocol for obtaining angiostatin formulations which may be used in clinical human trials.

Definitions

Definitions for other terms used herein are as follows. The terms "a", "an" and "the" as used herein are defined to mean "one or more" and include the plural unless the context is inappropriate. As used herein, the terms "detecting" or "detection" refer to qualitatively or quantitatively determining the presence of a molecule under investigation.

"Proteins", "peptides", "polypeptides" and "oligopeptides" are chains of amino acids (typically L-amino acids) whose alpha carbons are linked through peptide bonds formed by a condensation reaction between the carboxyl group of the alpha carbon of one amino acid and the amino group of the alpha carbon of another amino acid. The terminal amino acid at one end of the chain (i.e., the amino terminal) has a free amino group, while the terminal amino acid at the other end of the chain (i.e., the carboxy terminal) has a free carboxyl group. As such, the term "amino terminus" (abbreviated N-terminus) refers to the free alpha-amino group on the amino acid at the amino terminal of the peptide, or to the alpha-amino group (imino group when participating in a peptide bond) of an amino acid at any other location within the peptide. Similarly, the term "carboxy terminus" (abbreviated C-terminus) refers to the free carboxyl group on the amino acid at the carboxy terminus of a peptide, or to the carboxyl group of an amino acid at any other location within the peptide.

Typically, the amino acids making up a peptide are numbered in order, starting at the amino terminal and increasing in the direction toward the carboxy terminal of the peptide. Thus, when one amino acid is said to "follow" another, that amino acid is positioned closer to the carboxy terminal of the peptide than the preceding amino acid.

The term "residue" is used herein to refer to an amino acid (D or L) or an amino acid mimetic that is incorporated into a peptide by an amide bond. As such, the amino acid may be a naturally occurring amino acid or, unless otherwise limited, may encompass known analogs of natural amino acids that function in a manner similar to the naturally occurring amino acids (i.e., amino acid mimetics). Moreover, an amide bond mimetic includes peptide backbone modifications well known to those skilled in the art.

The term "angiostatin protein" refers to proteins that may be synthesized and may be isolated from biological tissues, cells, cell culture medium, and from broth and media obtained from cellular and cell-free expression systems. Accordingly, the term angiostatin protein includes angiostatin protein produced from recombinant expression systems. The term angiostatin protein also includes precursor forms of the angiostatin protein. The term angiostatin protein also includes fragments of the protein, and modified proteins and peptides thereof that have a substantially similar amino acid sequence, and that are capable of inhibiting proliferation of proliferation of blood vessels. For example, silent substitutions of amino acids, wherein the replacement of an amino acid with a structurally or chemically similar amino acid does not significantly alter the structure, conformation or activity of the protein, are well known in the art. Such silent substitutions are intended to fall within the scope of the present invention. The term angiostatin protein also includes various post-translational modifications or other modifications of angiostatin protein, including, but not limited to, phosphorylation, glycosylation, sulfation, and disulfide bond formation or reduction.

It will be appreciated that the term angiostatin protein, as used herein, includes shortened proteins or peptide fragments of angiostatin protein wherein one or more amino acids, preferably 1 to 10 amino acids, are removed from either or both ends of angiostatin protein, or from an internal region of the protein, yet the resulting molecule retains bioactivity such as inhibiting proliferation of blood vessels. The term angiostatin protein also includes lengthened proteins or peptides wherein one or more amino acids, preferably 1 to 10 amino acids, is added to either or both ends of angiostatin protein, or to an internal location in the angiostatin protein, yet the resulting molecule retains the ability to inhibit proliferation of blood vessels.

Also included in the definition of the term angiostatin protein are modifications of the angiostatin protein, its subunits and peptide fragments. Such modifications include substitutions of naturally occurring amino acids at specific sites with other molecules, including but not limited to naturally and non-naturally occurring amino acids. Such substitutions may modify the bioactivity of angiostatin protein and produce biological or pharmacological agonists or antagonists. Such substitutions may include conservative substitutions known to one of skill in the art, such as valine for alanine. Acceptable substitutions may also include modifications of amino acids, such as norleucine for leucine. It is to be understood that substitution of D amino acids for L amino acids is encompassed within the scope of the present invention. Some substitutions are described in *Dictionary of Biochemistry and Molecular* Biology, 2"a ed., J. Stenesh, John Wiley & Sons, 1989, the entirety of which is incorporated herein by reference. Additional modifications include addition of an amino acid, such as a tyrosine or another amino acid at specific locations in angiostatin protein or fragments thereof to enhance labeling potential with radioactive and non-radioactive labels, addition of molecules such as ricin, addition of radioactive and/or nonradioactive labels.

"Substantial sequence homology" means at least approximately 70% homology between the acid residue sequence in the angiostatin protein

analog sequence and that of angiostatin protein, preferably at least approximately 80% homology, more preferably at least approximately 90% homology.

Furthermore, one of skill in the art will recognize that individual substitutions, deletions or additions in the amino acid sequence of angiostatin protein, or in the nucleotide sequence encoding for the amino acids in the angiostatin protein, which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are conservatively modified variations, wherein the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);
2) Aspartic acid (D), Glutamic acid (E);
3) Asparagine (N), Glutamine (Q;
4) Arginine (R), Lysine (K);
5) Isoleucine (I), Leucine (L), Methionine (M); Valine (V); and
6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

Methods of Producing Angiostatin Protein

Angiostatin protein can be isolated from biological sources, including tissues, cells and biological fluids. Angiostatin protein may be produced from recombinant sources, from genetically altered cells implanted into animals, from tumors, and from cell cultures, as well as other sources. Angiostatin protein can be isolated from body fluids including, but not limited to, serum, urine and ascites, or synthesized by chemical or biological methods (e.g. cell culture, recombinant gene expression, cellular and cell free expression systems, peptide synthesis, and *in vitro* and in vivo enzymatic catalysis of precursor molecules to yield active angiostatin protein). Recombinant techniques include gene amplification from DNA sources using the polymerase chain

reaction (PCR), and gene amplification from RNA sources using reverse transcriptase/PCR.

Angiostatin protein can be made by automated protein synthesis methodologies well known to one skilled in the art. Alternatively, angiostatin protein may be isolated from larger known proteins. Angiostatin protein can also be produced synthetically by chemical reaction or by recombinant techniques in conjunction with expression systems.

It is contemplated as part of the present invention that angiostatin protein can be isolated from a body fluid such as blood or urine of patients. Angiostatin protein can also be produced by recombinant DNA methods or synthetic peptide chemical methods that are well known to those of ordinary skill in the art. In a preferred embodiment of the present invention, angiostatin protein is recombinantly produced. A preferred method of recombinant production of angiostatin protein is a method employing Pichia pastoris. Novel methods of isolation and purification of angiostatin protein, especially recombinantly-produced angiostatin protein are provided in the present invention.

It is to be understood that a variety of expression systems may be used for recombinant production of angiostatin protein. These expression systems include, but are not limited to Pichia pastoris, yeast, E. coli, insect cells, baculovirus expressions systems, expression in transgenic animals, expression in transgenic plants, mammalian systems, and other systems commonly known to one of ordinary skill in the art of expressing proteins. Some of these expression systems are described in U.S. Patent No. 5,854,205. Although the Pichia pastoris expression system was used for most of the recombinant angiostatin protein production presented in the present application, it is to be understood that the present invention encompasses other systems for recombinant production of angiostatin protein. Accordingly, modifications of the angiostatin protein production parameters presented herein can be made by one of ordinary skill in the art of recombinant production of proteins using specific expression systems. For example, when yeast are used for recombinant production of angiostatin

protein, different induction methods may be used, as commonly known to one of skill in the art. Yeast can be induced on methanol, or a mixture of methanol and glycerol, all optionally diluted with water, at feed rates commonly known to one of ordinary skill in the use of yeast expression systems for recombinant production of molecules, including proteins.

Culture Conditions

The following description of a preferred embodiment of the culture conditions for angiostatin protein is not limiting to the invention, and it is to be understood that the conditions, described here and in the examples may be scaled up or down to accommodate higher or lower requirements for production of angiostatin protein. These conditions may be scaled up to accommodate angiostatin protein production by 5, 10, 20 or 100 fold. It is also to be understood that the various concentrations of solutions and reagents described herein, including description contained in the Tables) are not limiting and may be increased or decreased in a range of 0 to 20%, preferably 0 to 10%, without altering the spirit and scope of the present invention.

Seed Culture

Inoculum cultures are prepared using a two stage seed process of *Pichia pastoris*. The first stage employs an enriched media (about 800mL in a 2.81, flask) and is incubated at 250 rpm and about 30°C for approximately 24 hours to a final optical density at 600 nm ($OD_{600nm,}$) of greater than 30. The second stage uses a similar media base (16 x 1L in 2.8L shaker flasks) and is incubated at about 250 rpm and 30°C for approximately 16 hours to a final OD_{600nm} of between about 20.0 and 30.0.

Main Fermentation

The fermentation media consists of Calcium Sulfate, Potassium Sulfate, Magnesium Sulfate, Potassium Hydroxide, Phosphoric Acid and Glycerol. Post sterilization addition of Trace Salts Solution is necessary. The fermentation

consists of four main phases; batch glycerol, fed-batch glycerol, methanol ramp and methanol soak.

The batch glycerol phase is the beginning phase which utilizes the initial charge of Glycerol as the carbon source. This phase lasts for approximately 30 hours. A sharp DO spike characterizes the end of this phase. The spike indicates the depletion of the carbon source.

The fed-batch glycerol phase is initiated at a set flow (16.1 g/Kg/hr) immediately following the batch glycerol phase. The fed-batch glycerol phase lasts for 6 hours. During the final two hours of the fed-batch phase, the pH is allowed to decrease from 5.0 to 4.0. The temperature is also decreased from 30°C to 26°C during the last two hours of the phase.

The methanol ramp phase is initiated immediately following the fed-batch glycerol phase. The methanol is used as a carbon source and as a product inducer. Angiostatin is produced as a secreted protein. During this phase, the methanol flowrate to the fermentor is ramped linearly from 1.5 to 4.5 mL/Kg/hr at a rate of 1.0 mL/Kg/hr^2.

The final phase of the fermentation is the methanol induction phase. The methanol continues to be used as a carbon source and product inducer. During this phase the methanol is fed to the fermentor at a set rate of 4.5mL/Kg/hr for ~83 hours. Harvest conditions are then set, after the conditions have been achieved the fermentation process is ready for harvest. To minimize foaming, the methanol and pH loops are not shutoff until the temperature is below 20°C. Final angiostatin concentration is approximately 500mg/L in the supernatant. The final WCW is approximately 300g/L.

Many of the solutions and other conditions used in the incubation are shown in the following tables. It is to be understood that these conditions are not limiting, and that they may be increased or decreased to accommodate scale up or scale down of the procedure to attain a desired production level of angiostatin protein.

Process Data Sheets

Fermentation

Seed

Culture

1st Stage

Media	Biotin	0.4 mg/L
Components/Concentra	Glycerol	10 g/L
tion:	Yeast Nitrogen Base	13.4 g/L
	Peptone	20 g/L
	Yeast Extract	10 g/L
	Potassium Phosphate Monobasic	10.9 g/L
	Potassium Phosphate Dibasic	3.5 g/L
Shake Volume:	800 mL	
Inoculum Size:	1.0mL	
Incubation Conditions:	250 rpm and 30°C	
Incubation End	OD600nm 20 - 30 (approximately 24 hours)	
Conditions:		

2nd Stage

Media	Biotin	0.4 mg/L
Components/Concentra	Glycerol	10 g/L
tion:	Yeast Nitrogen Base	13.4 g/L
	Potassium Phosphate Monobasic	10.9 g/L
	Potassium Phosphate Dibasic	3.5 g/L
Shake Volume:	16 x 1L	
Inoculum Size:	Such that initial OD600nm = 0.85 ± 0.15	
Incubation Conditions:	250 rpm and 30°C	
Incubation End	OD600nm 6.0 –12.0	
Conditions:	(approximately 16 hours)	
Testing:	Non-host Contamination	

Fermentation

Pre Inoculum

Specifications

Media	Calcium Sulfate, dihydrate	0.93 g/L
Components/Concentra	($CaSO_4*2H_2O$)	18.2 g/L
tion:	Potassium Sulfate (K_2SO_4)	14.9 g/L
	Magnesium Sulfate ($MgSO_4*7H_2O$)	4.13 g/L
	Potassium Hydroxide (KOH)	26.7 mL/L
	Phosphoric Acid (H_3PO_4)	40 mL/L
Density:	Glycerol	
Pre SIP Volume:	1.05 Kg/L	
Post SIP Volume:	900L (945Kg)	
(Calculations based on	927L (973Kg)	
Post SIP Volume):		
Sterile Additions:	PTM_4 Trace Salts	4.0 mL/L
Antifoam:	KFO 880 ~ 2L required	
Testing:	Media Hold (sterility)	

PTM_4 Solution

Component and	Cupric Sulfate ($CuSO_4*5H_2O$)	2 g/L
Concentration:		
	Sodium Iodide (NaI)	0.08 g/L
	Manganese Sulfate ($MnSO_4*H_2O$)	3 g/L
	Sodium Molybdate ($Na_2MoO_4*2H_2O$)	0.2 g/L
	Boric Acid (H_3BO_3)	0.02 g/L
	Cobalt Chloride ($CoCl_2*6H_2O$)	0.5 g/L
	Zinc Chloride ($ZnCl_2$)	7 g/L
	Ferric Sulfate ($FeSO_4*7H_2O$)	22 g/L
	d-Biotin	0.2 g/L
	Sulfuric Acid (H_2SO_4)	1 ML/L

Batch Glycerol Phase and Fermentation Conditions

Carbon Source:	Initial Charge of Glycerol
Temperature:	30°C
pH:	5.0
Agitation:	250 rpm
Aeration:	0.666 vvm (per Post SIP weight) = 650slpm
DO:	30% (controlled by oxygen supplementation)
Back-pressure:	3 psig
In Process Testing:	Wet Cell Weight, OD600nm, offline pH, and methanol concentration

Fed-Batch Glycerol Phase

Purpose:	Expansion of cell density
Start:	Approximately fermentation hour 30
Flowrate:	16.6 g/Kg/hr (per Kg of Post SIP weight)
Duration:	6 hours
Glycerol Specifics:	50% Glycerol Solution (by weight) with KFO 880 Antifoam (0.2 mL/Kg)
pH Shift Start:	4^{th} hour of Fed Batch Glycerol
pH Shift Specifics:	Linear Decrease from 5.0 to 4.0
pH Shift Duration:	2 hours
Temperature Shift Start:	4^{th} hour of Fed Batch Glycerol
Temperature Shift Specifics:	Linear Decrease from 30°C to 26°C
Temperature Shift Duration:	2 hours

Methanol Adaptation Phase	
Purpose:	Methanol as inducer and carbon source
Start:	Immediately following Fed-Batch Glycerol Phase
Initial Flowrate:	1.5 mL/Kg/hr (per Kg of Post SIP weight)
Final Flowrate:	4.5 mL/Kg/hr (per Kg of Post SIP weight)
Ramp Rate:	1.0 mL/Kg/hr2 (~3 hours)

Methanol Induction Phase	
Start:	Immediately following Methanol Ramp Phase
Flowrate:	4.5 mL/Kg/hr (per Kg of Post SIP weight)
Duration:	83 hours
Soak specifics:	Oxygen consumption ~ *Unknown at this time*
	slpm/Kg (per Kg of Post SIP weight)

Harvest Conditions and Specifics	
Temperature:	10°C
Agitation:	50 rpm (250 rpm until 20°C is reached)
Aeration:	50slpm (650 slpm until 20°C is reached)
DO Control:	OFF
Back-pressure:	3 psig
Specifics:	pH loop ON until <20°C is met
	Methanol flowrate reduced to 2.1 mL/Kg/hr,
	turned off when 20°C met
	Angiostatin Concentration ~0.5 mg/L in
	supernatant
	Final Weight of ~ 1450Kg
	Final WCW of ~ 300 g/L
QC Testing:	Non-Host Contamination

Method for Isolation and Purification of *Angiostatin Protein*

The present invention also provides a new and useful method for recovery and purification of proteins, particularly recombinantly-produced proteins. The methods of the present invention may be used for recovery and purification of angiostatin protein from biological sources, including but not limited to biological fluids, tissues, cells, culture media, and fermentation media. In one embodiment, the present invention provides a new and useful method for recovery and purification of angiostatin protein, and more particularly, recombinantly-produced angiostatin protein. This method may be employed for large scale recovery and purification of recombinantly-produced angiostatin protein. It is to be understood that the present invention is useful for recovery and purification of angiostatin protein from any expression system.

The basic recovery process of angiostatin protein is accomplished using four chromatography steps and a final concentration and diafiltration step. These steps are shown schematically in Figures 1 through 5. Figure 1 presents an overview of the process.

Upon completion of fermentation, the broth, which consists of all components (cells, nutrients, and buffer) within the fermenter, is diluted with water to a conductivity that favors binding of the target protein to the first column in the process.

The first chromatography step in the recovery and purification procedure is called the angiostatin protein purification capture step, and the specific resin used is called Streamline-SP (Pharmacia, Inc.). SP refers to the sulfopropyl functional groups that are attached to the support bead that give the resin its cationic character. It is to be understood that besides Streamline-SP resin, other resins that act as canon exchangers may be used in the practice of the present invention. Such cation exchangers include but are not limited to carboxymethylcellulose. Streamline refers to a relatively new format of chromatography that is designed to capture and separate target protein from a milieu of broth, thus eliminating the need for centrifugation to separate cells from the protein-containing supernatant. This type of chromatography is also known

as expanded bed absorption chromatography (EBA). In practice, the broth is typically pumped up into a Streamline column containing about 20-30% by volume of settled resin and approximately 70-80% buffer. As the broth enters the column, the bed of resin expends and flows up, thereby accounting for the name EBA. As the bed flows up, protein is bound to the beads, which can only flow up a finite distance, to an equilibrium level. The cells and non-bound protein however, flow up and out of the column to waste. Once all the broth has been pumped onto and traversed the column, the flow direction is reversed (now in the downward direction) and the resin is allowed to pack. What remains is a functional column that can be washed and eluted in the more conventional sense. Angiostatin protein is eluted from this column with salt, and is ready for the next chromatographic step.

The next chromatographic steps in the process are the Q-sepharose and hydroxyapatite chromatography columns. These steps are followed by the phenyl column as shown in the figures.

The final step in the purification procedure involved concentration and dialysis using the approach of Ultrafiltration/Diafiltration (UF/DF). In this step, the sample from the preceding step is pushed through a membrane, preferably made from polyethersulfone, with a molecular cutoff chosen to retain angiostatin protein or another protein of interest on the membrane. A preferred molecular cutoff for angiostatin protein is about 3kDa. Several liters of formulation buffer are run over the membrane to recover retain angiostatin protein, or another protein of interest remaining in the filters. This material recovered from the filters is added to the pool of angiostatin protein. In another embodiment of the present invention, parallel flow concentrators employing porous tubes may be used instead of flat membranes for concentration and dialysis.

The following paragraphs describe the chromatography steps in greater detail.

Streamline SP Chromatography

The expanded bed column (60cm x 18cm (settled height), 51L of Streamline SP Resin, expanded bed volume ~ 150L, expanded bed height of ~54cm at 300 cm/hr) is sanitized with 0.5M NaOH (held for a minimum of 6 hours). The column is rinsed with WPU until neutral conditions are met.

The column is equilibrated with 50mM Sodium Phosphate, 24mM Citric Acid, pH 5.1 until the pH and conductivity of the column are that of the buffer. The angiostatin Fermentation is loaded onto the column while performing inline dilution with WPU to maintain a load conductivity of 9-12 mS/cm. The column is washed with 15% Glycerol, 15mM Sodium Phosphate, pH 6.1.

The angiostatin is eluted from the column using 30mM Sodium Phosphate, 200mM NaCl, pH 7.2. Collection begins when the conductivity rises sharply (to > 4 mS/cm) and the UV rises above 0.5 AU. Collection ends when UV returns to 0.2 AU. The volume of the eluate should be approximately 2-3CV's at an angiostatin concentration of 3.3 g/L of eluate.

The column is regenerated using 7 column volumes (CV's) of 2M NaCl. The 2M NaCl Regeneration is followed with 6M Urea. After an initial Urea Wash, Urea is recirculated for a minimum of 1 hour. The Urea dissolves the cell paste and eases removal of the cell paste from the column. The regeneration is followed with a WPU flush until the UV returns to baseline.

The column is sanitized with 0.5M NaOH then stored in 0.1M NaOH. The 0.1 M NaOH may be prepared inline by mixing 0.5M NaOH and WPU so that the inlet conductivity is 23 ± 5mS/cm.

Q Sepharose FF and Ceramic Hydroxyapatite Chromatography

The Q-Sepharose column (30cm x 15cm column, 10.61, CV) and Ceramic Hydroxyapatite (CHT) column (45cm x 37cm column, 58.8L CV) which were stored in 0.1M NaOH are rinsed with 5 CV's of 10mM Sodium Phosphate, pH 7.0. The maximum flowrate for this chromatography is 480LPH (300 cm/hr of CHT Column) and is performed at ambient temperature. The angiostatin flows through the Q Sepharose column and binds to the CHT column. The columns are charged with 0.5M Sodium Phosphate, pH 7.0 then equilibrated with 10mM

Sodium Phosphate, pH 7.0 until the pH and conductivity are that of the equilibration buffer. The elution from the Streamline SP Chromatography of Angiostatin is diluted inline with WFI (1 part elution: 3 part WFI) and loaded onto the column. The column is washed to baseline with 10mM Sodium Phosphate, pH 7.0. The Q Sepharose column is removed from the chromatography skid.

The angiostatin is eluted from the CHT column with a 5CV linear gradient from 10mM Sodium Phosphate, pH 7.0 to 74mM Sodium Phosphate, pH 7.0. The 74mM Sodium Phosphate, pH 7.0 is continued until the UV returns to <0.5 AU. The product is collected from peak beginning at 0.15 AU to peak ending at 0.3 AU. The volume of the elution should be approximately 6-8 CV's at an angiostatin concentration of ~0.8g/L.

The CHT column is regenerated with 0.5M Sodium Phosphate, pH 7.0. The Q Sepharose FF column is regenerated with 2M NaCl. The columns are then cleaned with 0.5M NaOH and held for at least 1 hour (maximum of 24 hours). The columns are then stored in 0.1M NaOH which is prepared by blending 0.5M NaOH and WFI.

Toyopearl Phenyl 650M Chromatography

The Toyopearl Phenyl 650M Column (45cm x 25cm column, 40L CV) which was stored in 0.1M NaOH is rinsed with WFI until neutral conditions have been met. The flowrate for this chromatography is 480LPH (300cm/hr) and is performed at ambient temperature. The column is equilibrated with 50mM Sodium Phosphate, 24mM Citric Acid, 1.4M Ammonium Sulfate, pH 5.1 until the pH and conductivity are that of the equilibration buffer.

The elution from the CHT column is diluted inline with 50mM Sodium Phosphate, 24mM Citric Acid, 2.8M Ammonium Sulfate, pH 4.5 (1 part elution: 1 part buffer) and loaded onto the Toyopearl 650M column. The loaded column is then washed with 50mM Sodium Phosphate, 24mM Citric Acid, 1.4M Ammonium Sulfate, pH 5.1. The angiostatin is eluted from the column using a 20CV linear gradient from 50mM Sodium Phosphate, 24mM Citric Acid, 1.4M

Ammonium Sulfate, pH 5.1 to 50mM Sodium Phosphate, 24mM Citric Acid, 0.92M Ammonium Sulfate, pH 5.1. The 50mM Sodium Phosphate, 24mM Citric Acid, 0.92M Ammonium Sulfate is continued until UV returns to < 0.1 AU. The eluate is collected from peak beginning at 0.3 AU to peak ending at 0.1 AU. The elution volume is approximately 8CV's at an angiostatin concentration of ~ 0.7g/L. The column is regenerated with 50mM Sodium Phosphate, 24mM Citric Acid, pH 5.1. The column is rinsed with WFI and then cleaned with 0.5M NaOH. The column is then stored in 0.1M NaOH which. may be prepared by blending 0.5M NaOH inline with WFI. Note: If the Toyopearl Phenyl Elution will not be processed within 8 hours, the elution is to be diluted lx with WFI and stored at 2-8°C for a maximum of 48 hours.

The Ultrafiltration/Diafiltration steps are described in greater detail in the following paragraphs.

100sq. feet of 5Kd polyethersulfone filters are sanitized with 0.5M NaOH and held in 0.5M NaOH for a minimum of 1 hour (maximum of 2 hours). The filters are then rinsed with WFI until neutral conditions are obtained. The filters are then equilibrated with 0.15M Sodium Chloride until the retentate pH and conductivity is that of the equilibration buffer. The Toyopearl Elution (if not diluted) is diafiltered 1x with 0.15M Sodium Chloride. The Diafiltered product is concentrated to 5 mg/mL then diafiltered again until the pH and conductivity is that of the formulation buffer (~7 volumes). The UF/DF skid is rinsed with 2 x 10L flushes which are added to the diafiltered product. Due to the hold up volume of the UF/DF skid, it is necessary to perform the final concentration on a table top unit with 25sq. feet of filter. The retentate is then concentrated to 20.0 mg/mL. The UF/DF filters are rinsed with 0.15M Sodium Chloride and the rinse is added to the concentrated product. The UF/DF retentate is adjusted with 0.15M Sodium Chloride to a final concentration of 15 mg/ml. Note: If the Toyopearl Elution was diluted the lx diafiltration may be omitted.

Formulation

The formulated pool is then aseptically filtered through a 0.2micron filter. The filtered angiostatin is bulk filled into sterile bottles and then stored at -70°C.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. These conditions for angiostatin protein production and/or purification may be scaled up, for example, by 5, 10, 20 or 100 fold to accommodate the need for large scale angiostatin protein production. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof. which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention.

EXAMPLE 1

Sequence of Human Angiostatin Protein

The following is an example of a functional human angiostatin protein of the present invention. This is the angiostatin protein amino acid sequence encoded by the gene sequence listed below as SEQ ID NO: 1.

ANGIOSTATIN Production Clone ENMA98: Angiostatin gene and protein sequences. SEQ ID NO:1 shows the amino acid sequence of the angiostatin protein produced from the production clone ENMA98 which contains the nucleotide sequence shown in SEQ ID NO:2

hASv3 protein sequence (260 aa) SEQ ID NO: 1

VYLSECKTGNGKNYRGTMSKTKNGITCQKWSSTSPHRPRFSPATHPSE
GLEENYCRNPDNDPQGPWCYTTDPEKRYDYCDILECEEECMHCSGEN
YDGKISKTMSGLECQAWDSQSPHAHGYIPSKFPNKNLKKNYCRNPDR
ELRPWCFTTDPNKRWELCDIPRCTTPPPSSGPTYQCLKGTGENYRGNV
AVTVSGHTCQHWSAQTPHTHERTPENFPCKNLDENYCRNPDGKRAP
WCHTTNSQVRWEYCKIPSCDSSPV

hASv3 DNA sequence (780 bp) SEQ ID NO:2

GTGTATCTCTCAGAGTGCAAGACTGGGAATGGAAAGAATTACAGA
GGGACGATGTCCAAAACAAAAAATGGCATCACCTGTCAAAAATGG
AGTTCCACTTCTCCCCACAGACCTAGATTCTCACCTGCTACACACC
CCTCAGAGGGACTGGAGGAGAACTACTGCAGGAATCCAGACAACG
ATCCGCAGGGGCCCTGGTGCTATACTACTGATCCAGAAAAGAGAT
ATGACTACTGCGACATTCTTGAGTGTGAAGAGGAATGTATGCATTG
CAGTGGAGAAAACTATGACGGCAAAATTTCCAAGACCATGTCTGG
ACTGGAATGCCAGGCCTGGGACTCTCAGAGCCCACACGCTCATGG
ATACATTCCTTCCAAATTTCCAAACAAGAACCTGAAGAAGAATTAC
TGTCGTAACCCCGATAGGGAGCTGCGGCCTTGGTGTTTCACCACCG
ACCCCAACAAGCGCTGGGAACTTTGTGACATCCCCCGCTGCACAA
CACCTCCACCATCTTCTGGTCCCACCTACCAGTGTCTGAAGGGAAC
AGGTGAAAACTATCGCGGGAATGTGGCTGTTACCGTGTCCGGGCA
CACCTGTCAGCACTGGAGTGCACAGACCCCTCACACACATGAAAG
GACACCAGAAA.ACTTCCCCTGCAAAAATTTGGATGAAAACTACTG
CCGCAATCCTGACGGAAAAAGGGCCCCATGGTGCCATACAACCAA
CAGCCAAGTGCGGTGGGAGTACTGTAAGATACCGTCCTGTGACTC
CTCCCCAGTA

There is only one amino acid change (Asn-->Glu) in the protein sequence comparing to the wild type plasminogen K1-3, and one codon change in the nucleotide sequence (AAC-->GAA) corresponding to angiostatin protein.

EXAMPLE 2

Details concerning Chromatography, DiafiltrationlUltrafiltration, Bulk Formulation and Various Buffers used in the Procedure for Purification of ANGIOSTATIN Protein

Streamline SP Chromatography

Column

Specifications

Resin:	Streamline SP
Type:	Expanded Bed Adsorption
Particle Size:	200micron
Dimensions:	60cm x 18cm, 51L Resin, expanded bed volume ~ 150L, expanded bed height of ~54cm at 848 LPH
Pressure:	2.0 bar
Limitation:	
Expected Flowrate:	848 LPH (660 - 740 LPH for load and wash)

Sanitization and Rinse

Buffer:	0.5M NaOH
Approximate Volume Required	7CV (357L)
Flow Direction:	Up
Hold Time:	Minimum of 6 hours (maximum of 24 hours)
WPU Rinse:	Until conductivity < 1.0 mS/cm

Equilibration

Buffer:	50mM Sodium Phosphate, 24mM Citric Acid, pH 5.1
Approximate Volume Required:	14CV (714L)
Flow Direction:	Up
Equilibration Specifics:	Conductivity 5.5 - 6.5mS/cm PH = 5.1 ± 0.2

Load

Capacity:	> *unknown at this time* mg/mL
Load Conductivity:	9 - 12 mS/cm
WFI Dilution:	~3 volumes of WPU
Flow Direction:	Up
Loading Time:	~5.5 hours
Volume of Load:	~4500L

Wash

Buffer:	15% Glycerol, 15mM Sodium Phosphate, pH 6.1
Approximate Volume Required:	14CV (714L)
Flow Direction:	Up then down (12cv up / 2cv down)
Wash Specifics:	Conductivity < 1.5 mS/cm pH = 6.1 ± 0.2

Elution

Type:	Step Elution
Buffer:	30mM Sodium Phosphate, 200mM NaCl, pH 7.2.
Approximate Volume Required:	11CV (561L)
Flow Direction:	Down
Elution Specifics:	Collection begins UV > 0.5AU and Conductivity > 6 mS/cm Angiostatin Concentration 2.5 - 3.3 g/L
Approximate Eluate Volume:	2-3 CV
Product Storage Conditions:	Ambient (< 8 hours) 2-8°C (< 24 hours)
Product Testing:	LAL and UV

Regeneration #1

Buffer:	2M NaC1
Approximate Volume Required:	7CV (357L)
Flow Direction:	Up

Regeneration #2 and Rinse

Buffer:	6M Urea
Approximate Volume Required:	10CV Total (510L)
Flow Direction:	Up for 8CV (408L). Recirculate down with 2CV (100L) for 60 minutes
WPU Rinse:	Until UV return to baseline

Cleaning

Buffer:	0.5M NaOH
Approximate Volume Required:	7CV (357L)
Flow Direction:	Up
Hold Time:	Minimum 1 hour (maximum of 24 hours)

Storage

Buffer:	0.1M NaOH (inline dilution 0.5M NaOH / WPU)
Approximate Volume Required:	7CV (357L total, 71L 0.5M NaOH)
Flow Direction:	Down

Purification

General Purification

Storage Conditions:	Storage >8 hours at 2-8°C No stability data has been generated. Therefore, storage time should be limited to less than 24 hours. Final product storage is -70°C,
Extinction Coefficient:	2.08
Shear Sensitivity:	Not Determined
Concentration Limit:	Not Determined
In process Testing	LAL, UV, PD and QC retains

Q-Sepharose and Ceramic Hydroxyapatite Chromatography

Column Specifications

Resin:	Q Sepharose FF (Pharmacia)
Type:	Ion Exchange (Flowthrough)
Particle Size:	90micron
Dimensions:	30 cm D x 15 cm H 1 O.GL CV
Pressure Limitation:	3.0 bar
Expected Flowrate:	480 LPH
Resin:	Ceramic Hydroxyapatite (Biorad)
Type:	Mixed Mode
Particle Size:	40micron
Dimensions:	45 cm D x 37 cm H 58L CV
Pressure Limitation:	2.5 bar
Expected Flowrate:	480 LPH

Rinse

Buffer:	l0mM Sodium Phosphate, pH 7.0
Specifics:	Rinse until conductivity < 3.0 mS/cm

Charge

Buffer:	O.5M Sodium Phosphate, pH 7.0
Approximate Volume Required:	3CV (175L)
Flow Direction:	Up

Equilibration

Buffer:	l0mM Sodium Phosphate, pH 7.0
Approximate Volume Required:	5 - 7CV (292 - 408L)
Flow Direction:	Up
Equilibration Specifics:	pH = 7.0 ± 0.1 Conductivity =1.0 - 1.6 mS/cm

Load

Capacity:	6 - 13 mg/mL
Conductivity:	4 - 6 mS/cm
WFI Dilution:	3 Volumes Inline
Flow Direction:	Up
Loading Time:	1.5 hr
Volume of Load:	600L (4x Streamline Elution)

Wash

Buffer:	l0mM Sodium Phosphate, pH 7.0
Approximate Volume Required:	3CV (175L)
Flow Direction:	Up
Wash Specifics:	pH = 7.0 ± 0.2

Elution (from CHT)

Type:	Linear Gradient 0 to 100% (A to B)
Buffer A:	10mM Sodium Phosphate, pH 7.0
Buffer B:	74mM Sodium Phosphate, pH 7.0
Approximate Volume Required:	SCV (239L) Hold in B for SCV (239L)
Flow Direction:	Up
Product Collection:	Start @ 0.15 AU pre Peak and end @ 0.3 AU post Peak
Elution Specifics: .	pH = 7.0 ± 0.2 Volume - 4-S CV Angiostatin concentration ~ 0.8 mg/mL

Regeneration of CHT Column

Buffer:	O.5M Sodium Phosphate, pH 7.0
Approximate Volume Required:	3CV (143L)
Flow Direction:	Down

Regeneration of Q Sepharose Column

Buffer:	2M NaCI
Approximate Volume Required:	3CV (32L)
Flow Direction:	Down

Cleaning (both columns)

Buffer:	O.5M NaOH
Approximate Volume Required:	SCV (292L)
Flow Direction:	Down
Hold Time:	Minimum 1 hour (Maximum of 24 hours)

Storage

Buffer:	0.1M NaOH
Approximate Volume Required:	3CV (175L)
Flow Direction:	Down

Toyopearl Phenyl 650M Chromatography

Toyopearl Phenyl 650M Column Specifications

Resin:	Toyopearl Phenyl 650M (TosoHaas)
Type:	Hydrophobic Interaction
Particle Size:	65micron
Dimensions:	45 cm D x 25 cm H 40L CV
Pressure Limitation:	2.5 bar
Expected Flowrate:	480 LPH

Rinse

WFI Rinse:	Until conductivity < 1.0 MS/cm
Rinse Specifics:	Perform a 3CV gradient from O.1M NaOH to WFI then continue rinsing
Flow Direction:	Up

Equilibration

Buffer:	50mM Sodium Phosphate, 24mM Citric Acid, 1.4M Ammonium Sulfate, pH 5.1
Transition Specific:	Perform a 3CV gradient from WFI to EQ buffer the continue with equilibration
Approximate Volume Required:	4.5CV (180L)
Flow Direction:	Up
Equilibration Conductivity 154 - 171 mS/cm	
Specifics:	Density= 1.10 Kg/L

Load

Capacity:	> 12 mg/mL
Conductivity:	154 - 171 mS/cm
Buffer Dilution:	lx (50mM Sodium Phosphate, 24mM Citric Acid, 2.8M Ammonium Sulfate, pH 4.6)
Volume Buffer Required:	~ 350L
Flow Direction:	Up
Loading Time:	1.25 hr
Volume of Load:	~ 700L

Wash

Buffer:	50mM Sodium Phosphate, 24mM Citric Acid, 1.4M Ammonium Sulfate, pH 5.1
Approximate Volume Required:	IOCV (400L)
Flow Direction:	Up
Wash Specifics:	Conductivity= 154 - 171 mS/cm pH = 5.1 ± 0.2

Elution

Type:	Linear Gradient from 0 to 100% (A to B), Hold in B for 5CV
Buffer A:	50mM Sodium Phosphate, 24mM Citric Acid, 1.4M Ammonium Sulfate, pH 5.1
Buffer B:	50mM Sodium Phosphate, 24mM Citric Acid, 0.92M Ammonium Sulfate, pH 5.1
Approximate Volume Required:	20CV (800L) Hold for 5CV(200L) B
Flow Direction:	Up
Product Collection:	Start @ 0.3 AU pre Peak and end @ 0.1 AU post Peak
Elution Specifics:	Eluate Volume --8 CV's Angiostatin Concentration - 0.6 g/L

Regeneration

Buffer:	50mM Sodium Phosphate, 24mM Citric Acid, pH 5.1
Approximate Volume Required:	3CV (120L)
Flow Direction:	Down
WFI Rinse:	~ 3CV

Cleaning

Buffer:	O.5M NaOH
Approximate Volume Required:	4CV (160L)
Flow Direction:	Down
Hold Time:	Minimum 1 hour (Maximum of 24 hours)

Storage

Buffer:	0.1 M NaOH
Approximate Volume Required:	5CV (200 L)
Flow Direction:	Down

UF/DF

Filter Specifications

Skid:	OF Skid (25 - 45 LPM flowrate) and Table Top Unit (5 - 10 LPM)
Membrane:	5Kd Polyethersulfone
Membrane area:	100 sq. ft. (Skid) 25 sq. ft. (Table Top)
Recirculation rate:	25 - 45 L/min (Skid) 5- 10 L/min (Table Top)
Expected Flux rate:	~ 10 L/min (Skid) ~ 1 L/min (Table Top)
Feed Pressure Range:	10 - 25 psi (Skid) 5 - 10 psi (Table Top)
TMP Range:	10 - 20 psi (Skid) 5 - 10 psi (Table Top)

Equilibration

Buffer:	0.15M Sodium Chloride
Approximate Volume Required:	500L
Equilibration Specifics:	Conductivity - 12 -14 mS/cm a 18-22°C

Initial Diafiltration

Buffer:	0.15M Sodium Chloride
Approximate Volume Required:	1 DV (400L)

Initial Concentration

Starting Concentration:	0.6 g/L
Approximate Starting Volume:	330 L
Intermediate Concentration:	5 g/L
Approximate Final Volume:	30L
Approximate Concentration Factor:	8

Final Diafiltration

Buffer:	0.15M Sodium Chloride
Diafiltration Specifics	Until pH and conductivity are that of the 0.15M Sodium Chloride

Approximate Volume Required:	7DV (280L)

Rinse of UF/DF Skid

Buffer:	0.15M Sodium Chloride
Approximate Volume Required:	2 x 1 OL Flushes
Washout Specifics:	Add both flushes to product tank

Final Concentration (Performed on Table Top Unit)

Starting Concentration:	3 g/L
Approximate Starting Volume:	45 L
Intermediate Concentration:	20 g/L
Approximate Final Volume:	10 L
Approximate Concentration Factor:	4

Rinse of Table Top Unit

Buffer:	O.15M Sodium Chloride
Approximate Volume Required:	<_ 31,
Washout specifics:	Transfer all of rinse to the concentrated product

Dilution (May not be required)

Initial Concentration:	15.4 mg/mL
Approximate Initial Volume:	13 L
Final Concentration:	15.0 mg/mL
Approximate Final Volume:	13.3 L
Buffer:	0.1 SM Sodium Chloride
Approximate Volume Required:	0.3 L

Angiostatin Formulated Bulk

Bulk Filling Specifics

Bottle Type:	PETG
Size:	1 L
Fill per bottle:	800mL
Total # of bottles:	25

Release Testing

Quality - Appearance	Clear, colorless to slightly pink
Quality - Osmolality	Report
Purity - Size Exclusion HPLC	Report
Strength – UV Absorbance	10 - 20 mg= mL (based on 1 mg/mL = 2.08 AU '@ 280nm)
Identity - SDS-Page (non-reduced)	Corresponds to reference
Safety - DNA Threshold	Report
Safety - LAL	≥ 0.50 EU/mg
Safety - Bioburden	≥ 10 CFU/mL

Buffer *Preparation*

General Buffer Preparation

Specifics:	Buffers made by volume 21 day expiration on all buffers (based on safety)
In Process Testing:	pH and Conductivity (measured @ 18-22°C) Density for Streamline Wash Buffer and Phenyl Buffers
QC Testing:	LAL and Bioburden

50mM Sodium Phosphate, 24mM Citric Acid, pH 5.1

Unit Description:	Op Streamline Equilibration and Toyopearl 650M Regeneration	
Component and Concentration:	Sodium Phosphate, Dibasic	13.4 g/L
	Citric Acid, Monohydrate	5.04 g/L
pH Adjustment	NaOH or HCl	TBD
Conductivity:	5 - 7 mS/cm	
pH:	4.9 - 5.3	
Density:	1.00 Kg/L	

30mM Sodium Phosphate, 200mM NaCl, pH 7.2

Unit Description:	Op Streamline Elution	
Component and Concentration:	Sodium Phosphate, Dibasic	6.40 g/L
	Sodium Phosphate, Monobasic	0.846 g/L
	Sodium Chloride	11.69 g/L
pH Adjustment	NaOH or HCl	TBD
Conductivity:	19-23mS/cm	
pH:	7.0 - 7.4	
Density:	1.00 Kg/L	

15% Glycerol, 15mM Sodium Phosphate, pH 6.1

Unit Description:	Op Streamline Wash	
Component and Concentration:	Glycerol	189 g/L (15% v/v)
	Sodium Phosphate, Dibasic	0.7 g/L
	Sodium Phosphate, Monobasic	2.08 g/L
pH Adjustment	NaOH or HCl	TBD
Conductivity:	< 1.5 mS/cm	
pH:	5.9 - 6.3	
Density:	~ 1.04 Kg/L	

10mM Sodium Phosphate, pH 7.0

Unit Description:	Op Q Sepharose and CHT Equilibration/Wash/Elution	
Component and Concentration:	Sodium Phosphate, Dibasic	1.63 g/L
	Sodium Phosphate, Monobasic	0.54 g/L
pH Adjustment	NaOH or HCl	TBD
Conductivity:	1.0 - 1.6 mS/cm	
pH:	6.9 - 7.1	
Density:	1.00 Kg/L	

74mM Sodium Phosphate, pH 7.0

Unit Description:	Op Q Sepharose and CHT Elution	
Component and Concentration:	Sodium Phosphate, Dibasic	12.1 g/L
	Sodium Phosphate, Monobasic	3.98 g/L
pH Adjustment	NaOH or HCl	TBD
Conductivity:	6.0 - 7.2 mS/cm	
pH:	6.9 - 7.1	
Density:	1.00 Kg/L	

0.5M Sodium Phosphate, pH 7.0

Unit Description:	Op Q Sepharose and CHT Regeneration	
Component and	Sodium Phosphate, Dibasic	81.8 g/L
Concentration:	Sodium Phosphate, Monobasic	26.9 g/L
pH Adjustment:	NaOH or Phosphoric Acid	TBD
Conductivity:	35 - 41 mS/cm @ 18-20°C	
pH:	6.9 - 7.1	
Density:	1.00 Kg/L	

50mM Sodium Phosphate, 24mM Citric Acid,

1.4M Ammonium Sulfate, pH S._1

Unit Description:	Op Toyopearl 650M Equilibration/Wash/Elution	
Component and		
Concentration:	Sodium Phosphate, Dibasic	13.4 g/L
	Citric Acid	5.04 g/L
	Ammonium Sulfate	185.0 g/L
pH Adjustment:	NaOH or HCl	
Conductivity:	206 - 228 mS/cm	
pH:	4.9 - 5.3	
Density:	1.092 - 1.112 Kg/L	

50mM Sodium Phosphate, 24mM Citric Acid,

0.92M Ammonium Sulfate, pH 5.1

Unit Description:	Op. Toyopearl 650M Elution	
Component and	Sodium Phosphate, Dibasic	13.4 g/L
Concentration:		
	Citric Acid, Monohydrate	5.04 g/L
	Ammonium Sulfate	121.6 g/L
pH Adjustment:	NaOH or HCl	TBD
Conductivity:	156 - 173 mS/cm	
pH:	4.9 - 5.3	
Density:	1.068 - 1.084 g/L	

50mM Sodium Phosphate, 24mM Citric Acid,

2.8M Ammonium Sulfate, pH 4.5

Unit Description:	Op Toyopearl 650M Load Dilution	
Component and	Sodium Phosphate, Dibasic	13.4 g/L
Concentration:		

	Citric Acid, Monohydrate	5.04 g/L
	Ammonium Sulfate	370 g/L
pH Adjustment:	NaOH or HCl	TBD
Conductivity:	359 - 357 mS/cm	
pH:	4.3 - 4.7	
Density:	1.167 - 1.204 Kg/L	

Formulation Buffer (0.15M NaCl)

Unit Op Description:	O/DF Diafiltration Buffer
Component and Concentration:	Sodium Chloride 8.76 g/L
Conductivity:	12 - 14 mS/cm
Density:	1.00 Kg/L

Example 3

Biochemical characterization verified the identity of the purified protein as human ANGIOSTATIN® and indicated that the protein was over 95% pure. The initial step in purification, hydrophobic interaction chromatography (HIC), removed the majority of pigments and extraneous proteins; yielding 80-90% pure ANGIOSTATIN©. A number of resins and buffer systems were examined for ANGIOSTATIN© binding capacity and specificity. The binding capacity of ANGIOSTATIN© protein to Toyopearl Phenyl 650m (TosoHaas) was 20-30% higher than the binding capacity of Phenyl Sepharose high sub (Pharmacia) in PBS containing 3.0 M NaCl [pH 7.4]. At pH 7.4, a buffer system utilizing sodium chloride dramatically increased the binding specificity of Phenyl 650m for ANGIOSTATIN© versus an Ammonium Sulfate buffer system.

Example 4

ANGIOSTATIN© Purity and Identification of Glycosylation Site

Purification of ANGIOSTATIN® protein results in one major species as well as a number of minor species which can be separated by reverse phase chromatography and SDS-PAGE. Western blot analysis using a polyclonal primary antibody indicated that all minor species were related to the major intact angiostatin protein. Electrospray ionization mass spectrometry of the reduced protein detected two major components whose deconvoluted spectra indicated masses of 29788 Da and 29951 Da., consistent with an intact angiostatin molecule and an intact molecule with a single hexose sugar (+ 163 Da), respectively. Digestion of the molecule with lysyl endopeptidase followed by LC-MS indicated this modification is localized to Kringle domain 1 (residues 30-74). Analysis of a trypsin subdigest of this peptide by LC/MS/MS demonstrated that this sugar is linked to Serine 31. This was confirmed by N-terminal sequencing. The peptide map and N-terminal sequencing were also useful in identifying an additional glycosylation site and minor cleavage products of the angiostatin molecule.

All patents, publications and abstracts cited above are incorporated herein by reference in their entirety.

The invention has been described in detail with particular reference to certain embodiments, but variations and modifications can be made without departing from the spirit and the scope of the present invention.